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13. ABSTRACT (Maximum 200 Words) Purpose. To determine whether the agonist activities of tamoxifen are exaggerated in hormone-resistant breast cancers. Scope. We proposed that coregulatory proteins influence the direction of transcription by antagonist-occupied steroid receptors. We screened for such proteins, and identified three novel cDNA fragments encoding peptides that interact with antagonist-bound PRs. The aims were to clone the complete cDNAs and define their structure (Aim 1); define the role of the unknown proteins on receptor activity (Aim 2); and, if appropriate, determine the role of these proteins in hormone dependency of breast cancers (Aim 3). Major Findings - Results. We have focused on one novel cDNA fragment, designated ORF#93. In the first year we cloned the full-length cDNA; assembled its genomic structure; localized the gene to chromosome 15q26.1; expressed the full-length protein; defined its tissue distribution; determined its subcellular localization to be cytoplasmic; and generated a polyclonal antibody that probes a 103 kDa protein. Functional studies have been started and are ongoing. The protein does not appear to have a major effect on transcription; antisense studies have been devised to test this further. Significance. We now believe that ORF#93 has a cytoplasmic "scaffolding" function, and allows receptors to interact with other proteins in multiprotein complexes, perhaps in association with hsp90. If so, ORF#93 may be important for cross-talk between growth factor and nuclear receptor signaling pathways.				
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INTRODUCTION

Subject. The antiestrogen tamoxifen is one of the most effective hormonal agents for treatment of estrogen (ER) and progesterone (PR) receptor-positive breast cancers. However, development of resistance to tamoxifen is common, and is a major obstacle to long-term treatment success. Tamoxifen, and the antiprogesterin RU486, are “mixed antagonists” having both agonist and antagonist properties. We have shown that the balance between agonist vs. antagonist transcriptional activities can be influenced by the abundance of nuclear receptor coregulatory proteins: the corepressors N-CoR and SMRT suppress the partial agonist activities of tamoxifen and RU486, while the coactivator L7/SPA enhances their partial agonist activity (Jackson et al. 1997). **Purpose.** However, these coregulators are unlikely to be the sole players in transcriptional regulation by ER and PR under the influence of antagonists. We proposed that the full complement of coregulatory proteins that could influence the direction of transcription by antagonist-occupied steroid receptors, had yet to be completely identified. L7/SPA is the only antagonist-specific coactivator defined to date. It is unlikely to be the only, or even the most important, protein to have this property. Similarly, it is unlikely that SMRT and N-CoR are the only corepressors that interact with antagonist-occupied steroid receptors. We believe that these three represent a minor subset of the whole, for two reasons. First, screening for coregulators has not focused on receptor N-termini, yet AF1 may be critical to the agonist effects of mixed antagonists (Webb et al. 1998). Newer experimental strategies may correct this deficiency (Aronheim et al. 1997; Norris et al. 1999). Second, recent crystallographic analyses show subtle structural variations in the conformation of the C-terminal ligand binding domain, dictated by the identity of the ligand (Shiau et al. 1998; Williams and Sigler 1998). For the most part, protein interaction screens for C-terminal binding proteins have not used antagonists. We proposed to use an antagonist-biased screen to identify true antagonist-specific coregulators. **Scope.** In preliminary studies, we identified three partial cDNA clones encoding proteins that specifically interacted with antagonist-bound PRs. We proposed to clone the complete cDNAs and define their structure (Aim 1); to define the role of the unknown proteins on receptor activity (Aim 2); and, if they look promising, to determine the role of these proteins in hormone dependency of breast cancers (Aim 3). In the first year we have focused on one novel cDNA fragment, designated ORF#93, whose features highlighted it as particularly interesting and relevant to our hypothesis. Therefore, the initial research effort has been focused on completely cloning and characterizing this gene and its protein product. The results obtained to date are outlined according to the headings of the original *Statement of Work* for this project.

BODY

Task 1: To clone, sequence and define the structure of three novel, antagonist-specific, ER and PR-interacting proteins (months 1-18)

Perform 5'-RACE to clone and sequence full length ORF#93

5'-RACE (rapid amplification of cDNA ends) was carried out using Clontech's SMART RACE technology. mRNA from HeLa cervicocarcinoma cells and T47D breast cancer cells, was reverse transcribed using an oligo (dT) primer. A universal primer was tethered to the 5'-end of the 1st strand cDNA by targeting the dCTP-rich tail added to the end of the newly synthesized strands by reverse transcriptase. This allowed 5'-targeted PCR amplification of the 1st strand cDNA using the universal forward primer and a gene-specific reverse primer. **Figure 1** indicates the location of the primers used, with respect to the most 5' ORF#93 clone originally retrieved from screening a λ gt11 HeLa cell cDNA library. This clone contained a 3.1kb ORF#93 cDNA but lacked the complete 5'-end of the gene. The following oligos were designed: two reverse primers, R1 and R2, that anneal to the 5'-end of the cDNA; an oligonucleotide probe, S1, for Southern blotting the subsequent RACE products; two primers, +F and +R, to anneal to the 3'-end of the cDNA and act as an internal control for the presence of the template in the RACE product; and a λ gt11 vector primer, gt11F, to use as a PCR control with the λ gt11 ORF#93 phage as template.

PCR amplification of RACE cDNA using the RACE forward primer and either R1 (Fig.1, lanes 1 and 6) or R2 (Fig.1, lanes 2 and 7) produced multiple faint bands on an ethidium bromide stained agarose gel. Using the same RACE cDNA template (Fig.1, lanes 3 and 8), the λ gt11 clone (Fig.1, lanes 5 and 10) or a plasmid vector containing the 3.1kb ORF#93 insert, the internal primers +F and +R produced a band of the expected size (1003bp). Primers gt11F and R2 produced the expected 230bp fragment when the λ gt11 clone was used as template (Fig.1, lanes 4 and 9). A no template control was completely negative with RACE forward primer and R2 (Fig.1, lane 11). The PCR products were Southern blotted, and probed using S1. As expected, the 230bp control fragment (lanes 4 and 9) strongly hybridized to the probe, whereas the 1003bp fragment was not detected. Strong bands were detected at 200 to 300bp in the RACE products amplified using RACE forward primer and R1 (lanes 1 and 5) or R2 (lanes 2 and 6). Primer R2 produced a band that was slightly larger than the band produced by R1, and the difference appeared similar to the distance between the start of the two primers (52bp). The fragments were subcloned and several clones were sequenced. Additional 5'-sequence of ORF#93 was obtained, with a potential start codon 62bp upstream of the existing clone. Further sequencing upstream of this ATG revealed an in frame stop codon at -114bp, respective to the new start site, suggesting that the full gene had been cloned. Using a unique KasI site at the 5'-end of ORF#93, the sequence identified by 5'-RACE was combined with the rest of the cDNA to make a full-length 3.3kb clone. **These experiments generated the entire protein coding sequence.**

At the same time as RACE cloning of the full ORF#93 cDNA was achieved, the sequence of a 156089bp genomic clone containing an unorganized series of 9 contigs was

made available on the high throughput genomic database (Accession code AC004886). **This information, together with the protein coding sequence, allowed us to determine the genomic structure of ORF#93.** The DNA is organized into 20 exons and is located on chromosome 15q26.1. The first intron (**Figure 2**) is 166bp and falls upstream of the 5'-end of the 3.1kb phage clone, and downstream of the first methionine and in-frame stop codon. This assignment was confirmed by primer-specific RT-PCR spanning the TGA and first ATG, since several potential splice sites were contained in this region. **These experiments defined the transcription start-site for the longest ORF#93 transcript.**

Analyze the ligand dependency and specificity of interactions between the three proteins, and PR and ER, using a mammalian two-hybrid system and GST pull-down assays

was combined with

Analyze the interactions of the three proteins with other members of the nuclear receptor family

The full-length ORF#93 cDNA was subcloned into pGEX 4T-1, downstream from the sequence encoding glutathione S-transferase, and a gst-93 fusion protein was produced in E.coli. The purified protein was bound to glutathione sepharose, and used in a series of gst pull-down experiments with PR-A or -B, glucocorticoid receptors (GR) or ER, all over-expressed in HeLa cells. Gst-93 interacted with PR-A, PR-B, and GR, whether unliganded or in the presence of agonist or antagonist (**Figure 3**). The strongest binding observed was with unliganded or antagonist bound receptors. Little or no binding was observed to gst alone, suggesting that the interaction was specific for gst-93. gst-93 also bound to ERs, and the specificity of this interaction is still being confirmed. **Thus protein 93 appears to interact with PR and GR either in the unliganded, or antagonist-occupied state.** Additional studies to confirm this are in progress.

Define the tissue distribution for the three receptor interacting proteins using human tissue RNA blots; their developmental expression using fetal mouse blots; and prepare polyclonal antibodies for subcellular localization studies.

The 3.1kb ORF#93 cDNA was labelled by ³²P-dCTP random priming, and used to probe a Clontech multi-tissue RNA master blot. The blot contained equal amounts of dot blotted RNA from 43 adult human tissues, seven fetal tissues, and 8 controls which included yeast, E.coli and human RNA and DNA. Trace amounts of ORF#93 transcript were detected in most tissues (**Figure 4**) suggesting that as with other coregulatory proteins, ORF#93 is ubiquitously expressed at limiting levels. **The strongest expression of the gene was seen in lung and kidney. Expression was also clearly detectable in a number of potential nuclear receptor targets, such as ovary, testis, adrenal, pituitary and mammary gland.** Interestingly, expression was much lower in fetal lung and kidney than in the adult, suggesting that ORF#93 plays a more important role in the adult. No hybridization of the probe was observed to the negative controls, except to E.coli and human DNA. Hybridization to E.coli DNA but not yeast DNA or yeast or E.coli RNA simply suggests some degree of homology to bacterial sequences. Hybridization to the human DNA control suggests three possibilities: the gene is highly expressed, or it contains repetitive sequence, or it is a member of a multi-gene family.

The last of these three is most likely since the transcript is not very abundant, and no hybridization of the probe was seen to the other repetitive sequence control (C₀t-1 DNA). This may be due to the **presence of three tetra-tricopeptide (TPR) repeat domains in the coding sequence**. Similar domains are found in other proteins, in a number of organisms. (TPR domains are discussed further, below). Therefore, while informative, these results suggest that it may be important to confirm this finding using a truncated cDNA probe lacking these TPR sequences.

A polyclonal antibody to ORF#93 was produced using a peptide sequence predicted to be antigenic from the total protein sequence. **The antibody detects recombinant, denatured ORF#93 on immunoblots. The protein has an apparent molecular weight of 103 kDa when transfected into mammalian cells (Figure 5).** The polyclonal does not recognize the folded native protein. Additionally, we have been unable to detect the endogenous protein in HeLa or T47D cells using this antibody, even under denaturing conditions, suggesting that **ORF#93 is present in limiting quantities in these cells.**

Perform RT-PCR using ORF#127 specific primers to clone and sequence full length ORF#127 cDNA

This task remains to be completed, as we have chosen to focus on ORF#93.

Task 2: to define the transcriptional coregulatory or other functional properties of the three receptor-interacting proteins (months 12-24)

Clone the three receptor interacting proteins into expression vectors

The full-length ORF#93 cDNA was cloned into two expression vectors: the pSG5 mammalian expression vector, for use in transcriptional studies, and the EGFP-C1 expression vector which fuses the protein C-terminal to the green fluorescent protein (gfp) cDNA, for use in protein localization studies.

Using transfection assays, determine whether ORF#61 (NIP7), ORF#93 and ORF#127 influence transcription induced by ER and PR in the presence of different ligands, and in several mammalian cell lines, including breast cancer cells

The ability of ORF#93 to influence nuclear receptor function was determined by transfection into mammalian cells, including HeLa cells and T47D-YB human breast cancer cells. **ORF#93 does not appear to act through modulation of receptor transcriptional activity.** In the experiment shown in **Figure 6**, HeLa cells were cotransfected with PR-B and a progestin-responsive reporter (pA3-PRE₂-TATA_{tk}-LUC) and increasing DNA amounts of ORF#93. In this experiment, a modest increase in activity was seen in the presence of the agonist R5020 with increasing amounts of ORF#93. This effect was not seen with RU486. Similar experiments have been carried out using GR or ER, and no agonist-specific stimulation was observed. Further transcriptional studies are being carried out to fully characterize any effect. We have constructed an antisense vector that knocks out expression of endogenous ORF#93, and are using this to define possible decrements in PR function in the absence of this protein.

Additionally, recent studies with the yeast proteins Ssn6 (another TPR domain protein) and corepressor Tup1 have shown that the two proteins act together to form the repressor complex (Gounalaki et al. 2000; Smith and Johnson 2000). Therefore, transcriptional studies incorporating the corepressors SMRT and N-CoR are being carried out to fully explore the possible transcriptional role of ORF#93.

Perform other functional studies with expression vectors for the three clones dictated by the structural analyses

As mentioned above, the ORF#93 cDNA was cloned into the EGFP-C1 expression vector to produce a gfp-93 fusion protein. This protein can be visualized in cells under fluorescent light. The construct was then expressed in T47D-YB cells (containing PR-B) and the cells were examined for localization of the protein in the presence or absence of PR ligands. Cells were treated for one hour with 10nM R5020, 100nM RU486 or ethanol vehicle control, then fixed using cold 30% methanol/70% acetone and nuclei were visualized using DAPI which stains the nuclei. DAPI staining is blue and gfp-93 is green. In the absence of ligand, **gfp-93 has a cytoplasmic localization (Figure 7)** and exhibits a punctate expression pattern, suggestive of golgi localization. The protein appears to be excluded from the nucleus. One hr. treatment of cells with progestin agonist or antagonist did not change the localization of gfp-93, although this is abundant time to see complete nuclear translocation of PR. This suggests that, **if ORF#93 interacts with PRs intracellularly, it does so in the cytoplasm.** This is consistent with what is known about TPR domain-containing proteins: these proteins act as chaperones and interact with other chaperonins such as hsp90 (Scheufler et al. 2000) – another protein that binds PRs in the cytoplasm. In order to address the possibility that ORF#93 interacts with PRs as part of a multi-protein complex that includes hsp90, co-immunoprecipitation experiments are being performed. **Preliminary results indicate that ORF#93 interacts with hsp90.** We are now testing the hypothesis that ORF#93 binds to nascent PRs, and facilitates their maturation and processing prior to nuclear transport.

ORF#93 mRNA expression and its progesterone regulation has also been examined. T47D-YB breast cancer cells were treated for 2 or 12 hours with 10nM R5020, 100nM RU486 or vehicle control. Cells were harvested and total RNA was isolated. 30µg of each sample was run on a denaturing agarose gel and Northern blotted using a ³²P-labeled ORF#93 cDNA probe. An apparent modest decrease in transcript expression, to 52% of the time matched control, was seen after 12h R5020 treatment (**Figure 8**). RU486 had little or no effect on transcript expression. **Thus, ORF#93 levels may be regulated by progesterone,** and its regulation by other steroid hormones is under investigation.

KEY RESEARCH ACCOMPLISHMENTS

- A full cDNA clone has been obtained for the novel DNA fragment designated ORF#93, which may be involved in nuclear receptor function.
- The genomic structure has been defined; the gene is located at 15q26.1.
- The full-length protein has been expressed.
- A direct protein-protein interaction between ORF#93 and PR, GR and ER, has been demonstrated in vitro.
- The expression pattern of ORF#93 has been described in adult and fetal human tissues.
- The influence of ORF#93 on nuclear receptor transcriptional activity has been characterized and is the subject of ongoing work.
- In vivo localization of the ORF#93 protein has been determined; the protein is cytoplasmic.
- A polyclonal antibody to ORF#93 has been generated and the size of the recombinant protein has been determined.

REPORTABLE OUTCOMES

The preliminary results generated by this project have been reported in the following meeting abstracts (appended):

Graham, JD, Abel, MG, Jackson, TA, Gordon, DF, Wood, WM, Horwitz, KB (2000)
Novel interactors mediating mixed antagonist action on estrogen and progesterone receptors in breast cancer. Proceedings of the Keystone Symposium: Nuclear Receptors 2000, USA, 2000.

Graham J D, Abel M G, Gordon D F, Wood W M and Horwitz K B (2000)
Receptor interacting proteins and the function of progesterone and estrogen receptors in breast cancer. Proceedings of the International Congress on Endocrinology 2000, Sydney, Australia, 2000.

The results summarized above are also being collated into a manuscript for imminent submission. At that time a copy will also be provided to USAMRMC.

CONCLUSIONS

This project seeks to identify novel proteins that interact with antagonist-occupied steroid receptors and modify the direction of transcription. Specifically, the studies are based on the hypothesis that "mixed antagonists" such as tamoxifen, exert either more or less agonist-like activity, depending on the nature of coregulatory proteins that are recruited to the transcription complex. In the first year of this grant, we have analyzed one cDNA fragment, dubbed ORF#93, that was isolated based on its ability to bind antagonist-occupied receptors. We have now cloned the full-length cDNA, defined its genomic structure, obtained a chromosome assignment, expressed the full-length protein, defined its tissue distribution and subcellular localization, and generated a polyclonal antibody that recognizes a 103 kDa protein. Functional studies have been started and are ongoing. The protein does not appear to have a major effect on transcription, but antisense studies have been devised to test this further. Instead, we now believe that the protein has a cytoplasmic "scaffolding" function, and serves as a matrix to allow PRs and other nuclear receptors to interact with other proteins in multiprotein complexes, perhaps in association with hsp90. If so, ORF#93 may be a key protein that brings PRs into close association with cytoplasmic signaling molecules with which these receptors would otherwise not interact. For example, we have shown in other studies, that PRs interact with STATs, yet these two proteins reside in different cellular compartments. Does ORF#93 function to bring these two disparate signaling molecules together? If so, ORF#93 may be important for cross-talk between growth factors and nuclear receptors, and we propose to test this hypothesis in the next series of studies.

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APPENDICES

Abstracts:

Presented at **Proceedings of the Keystone Symposium: Nuclear Receptors 2000, USA, 2000.**

Novel Interactors Mediating Mixed Antagonist Action on Estrogen and Progesterone Receptors in Breast Cancer.

J. Dinny Graham, M. Greg Abel, Twila A. Jackson, David F. Gordon, William M. Wood and Kathryn B. Horwitz. Division of Endocrinology, University of Colorado Hlth Sc Ctr, B151, 4200 E. 9th Ave, Denver, Colorado 80262, USA.

The antiestrogen tamoxifen is one of the most effective treatments for estrogen receptor (ER) positive breast cancer. However, tumors inevitably develop resistance to the treatment, which we postulated is due to the emergence of inappropriate agonist-like effects of this mixed antagonist. We have shown that the balance of agonist and antagonist activities of mixed antagonists is influenced by the abundance of nuclear receptor coregulators. We demonstrated that the corepressors N-CoR and SMRT, suppress the partial agonist activities of tamoxifen and the mixed antiprogesterin RU486 on ER and progesterone receptor (PR), respectively. Furthermore, a novel coactivator, L7/SPA, enhances partial agonist activity. These effects are mixed antagonist-specific, and are not observed with agonists or pure antagonists. In addition, we found that the expression levels of these coregulators may differ between tamoxifen sensitive and resistant breast tumors, suggesting that they may be determinants of tamoxifen responsiveness. We postulated that other novel factors may play a specific role in determining mixed antagonist effects in breast cancer. We have employed two different antagonist-specific screening strategies to identify proteins involved in tamoxifen and RU486 action. Conventional yeast 2-hybrid screening was performed in the presence of RU486, with the hinge and hormone binding domain of PR as bait. We have identified a novel protein of approximately 109 kDa, which interacts with PR only when liganded to RU486. The protein contains eight nuclear receptor (NR) binding LXXLL domains. Mutagenesis of one out of two NR boxes, contained in the original 2-hybrid clone, resulted in loss of PR interaction with that fragment. The protein also contains three putative tetratricopeptide repeat domains, which may be involved in nuclear targeting of RU486-liganded PR and act as a scaffold for assembly of PR into multiprotein complexes. Recent evidence suggests that mixed antagonist-specific interactions with ER and PR involve multiple contacts with both AF-1 and AF-2 of the intact receptors. To screen for such proteins we have used a Sos recruitment 2-hybrid screening strategy with a full length ER bait, in the presence of tamoxifen. A number of antagonist-specific ER interacting proteins have been isolated and will be described.

Presented at **Proceedings of the International Congress on Endocrinology 2000, Sydney, Australia, 2000.**

**RECEPTOR INTERACTING PROTEINS AND THE FUNCTION OF
PROGESTERONE AND ESTROGEN RECEPTORS IN BREAST CANCER**

Graham J D, Abel M G, Gordon D F, Wood W M and Horwitz K B

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The nuclear receptors for estrogen and progesterone (ER and PR) are important therapeutic determinants in breast cancer. Tumors expressing both receptors are generally well differentiated, indolent, and likely to respond to treatment with the mixed antiestrogen, tamoxifen. However, responsive tumors inevitably become tamoxifen-resistant and progress, often in the face of continued ER expression. We postulated that this is due to an increase in the partial agonist activity of tamoxifen. To test this hypothesis we have been searching for novel proteins that interact with receptors and modify the activities of mixed antagonists like tamoxifen. Using mixed antagonist-biased interaction screening, we have identified proteins that interact with ER and PR, and regulate transcription. The corepressors N-CoR and SMRT suppress the partial agonist activities of tamoxifen and the antiprogesterin RU486, whereas the coactivator L7/SPA enhances this activity, yet has no effect on pure agonists or antagonists. In tamoxifen-resistant tumors removed from patients, we see a trend towards decreased expression of corepressors. In the same screen we identified a cDNA fragment encoding a novel protein, that we have now cloned and fully sequenced. The 109 kD protein interacts best with unliganded and mixed antagonist-bound PR, and less well with agonist-bound PR. The 944 amino acid protein sequence contains four nuclear receptor interaction LXXLL motifs. Additionally, there are three tetratricopeptide repeat (TPR) motifs in the N-terminus, characteristic of chaperonin/immunophilin binding proteins. Indeed, hsp90 also interacts with the protein strongly in protein interaction experiments. When expressed as a green fluorescent fusion protein, it shows a punctate cytoplasmic localization, which persists in the presence of progestins. We are testing the hypothesis that this protein has a scaffolding function, and plays an integral role in the correct expression and folding of nascent receptors, and perhaps their subcellular localization.

Figures:

Figure 1: 5'-RACE-PCR Cloning of ORF#93

Rapid amplification of cDNA ends (RACE) was performed to clone the complete 5'-end of the ORF#93 gene.

- A. Location of primers used, with respect to the longest, most 5' cDNA clone obtained by screening a cDNA library.
- B. 1.2% agarose gel analysis of RACE products. Templates: lanes 1-3, 6-8, 11 - HeLa Race cDNA, lanes 4, 5, 9, 10 - λ gt11-ORF#93 clone (shown in A), lane 12 - plasmid DNA containing 3.1kb ORF#93 cDNA insert.
- C. Southern blotted gel (shown in B) probed with 32 P-labeled S1 oligonucleotide probe (shown in A)

Figure 2: Genomic organization of the 5'-region of the gene encoding ORF#93

The region of the ORF#93 gene, containing the first methionine (representing the translational start), and the upstream in-frame stop codon are indicated. The location of the first intron in relation to the original cDNA clone, RACE primers R1 and R2, and the unique upstream *KasI* site are also shown.

Figure 3: Interaction of ORF#93 with PR and GR

The ORF#93 cDNA was cloned C-terminal to the glutathione S-transferase (*gst*) sequence in pGEX-4T-1 and expressed in BL21 codon+ *E.coli*. Interaction between *gst*-93 and PR A, PR B or GR was detected by *gst* pull-down assay. Receptors were visualized by SDS-PAGE and immunoblotting. *Gst*, alone, was included as a control for non-specific interaction.

Figure 4: Tissue expression pattern of ORF#93 mRNA

- A - The ORF#93 cDNA was labelled and used to probe a multi-tissue dot blot. Results were visualized by phosphorimager.
- B - Results were quantitated densitometrically, and are shown as relative arbitrary densitometric units.

Figure 5: Expression of pSG5-ORF#93 in HeLa and COS cells

The full length ORF#93 cDNA was cloned into the pSG5 mammalian expression vector and co-transfected into HeLa and COS cells, with a β -galactosidase expression vector, to control for transfection efficiency. The cells were harvested, whole cell extracts were prepared and equal β -galactosidase amounts were run on a denaturing 7.5% polyacrylamide/SDS gel. Proteins were transferred to nitrocellulose membrane, and ORF#93 protein was detected using the polyclonal antibody.

Figure 6: Effect of ORF#93 on transcriptional activity of PR

The pSG5-ORF#93 mammalian expression vector was cotransfected into HeLa cells in increasing amounts, with PR and a progestin-responsive reporter (pA3-PRE₂-TATA_{tk}-LUC). Transfected cells were treated with 10nM R5020, 100nM RU486, or vehicle control, then harvested after 24h and lysed. Luciferase activity from the reporter was estimated using a luminometer. Relative luciferase activity is expressed as a percentage of the equivalent treated control, in the absence of ORF#93.

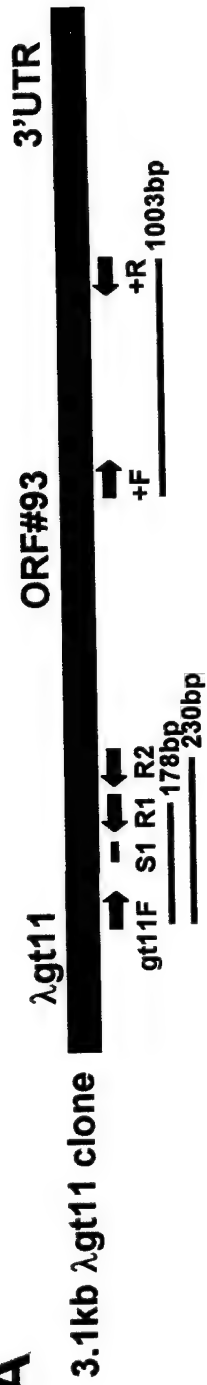
Figure 7: Localization of ORF#93

The full length ORF#93 cDNA was fused C-terminal to green fluorescent protein (gfp) cDNA in the EGFP-C1 mammalian expression vector. The gfp-93 protein was expressed in T47D-YB cells by transient transfection, and visualized under fluorescent light (green). Cell nuclei were visualized using DAPI stain (blue).

Figure 8: Regulation of ORF#93 expression by R5020 and RU486

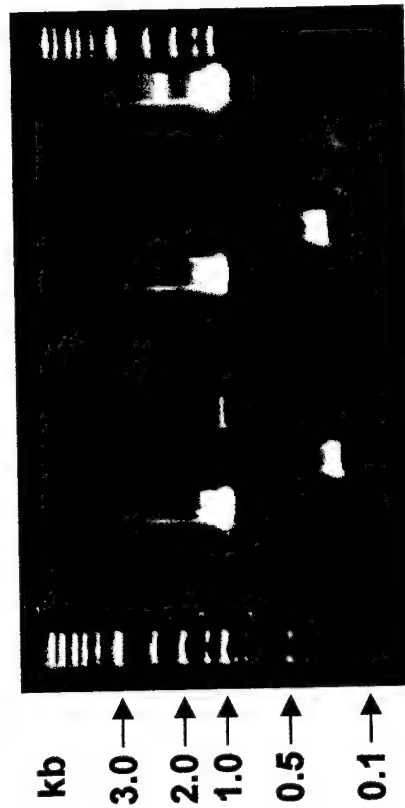
T-47D-YB cells growing in MEM medium containing charcoal stripped fetal bovine serum, were treated 2h and 12h with 10nM R5020, 100nM RU486 or vehicle control. Cells were harvested and total RNA was isolated by the guanidinium-cesium chloride method. Total RNA (30µg/lane) was analyzed by Northern blotting, using the 3.1kb ORF#93 cDNA as a probe. Expression of ORF#93 was estimated densitometrically.

A



B

	gt11F	RACE Fwd Primer	R1	R2	+F	+R
-	-	-	-	-	+	+
-	+	-	-	+	-	-
-	-	-	-	-	+	+
+	-	-	-	+	-	-
-	-	+	-	+	+	+
-	+	+	-	-	-	-
-	-	-	-	+	+	+
+	-	-	-	+	-	-
-	-	-	-	+	+	+
-	+	-	+	-	-	-
-	+	+	-	-	-	-



1 2 3 4 5 6 7 8 9 10 11 12

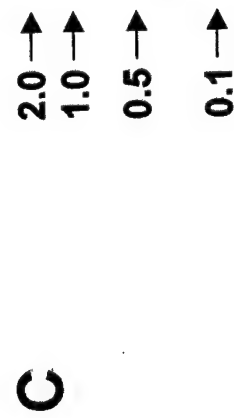


Figure 2

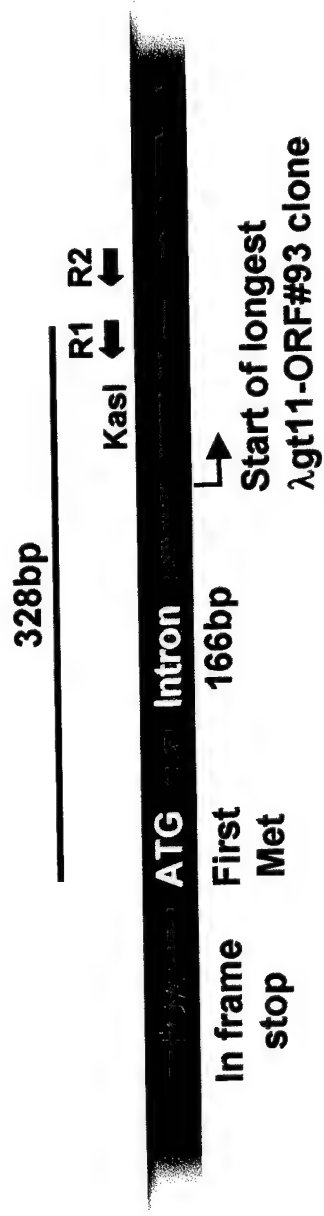


Figure 3

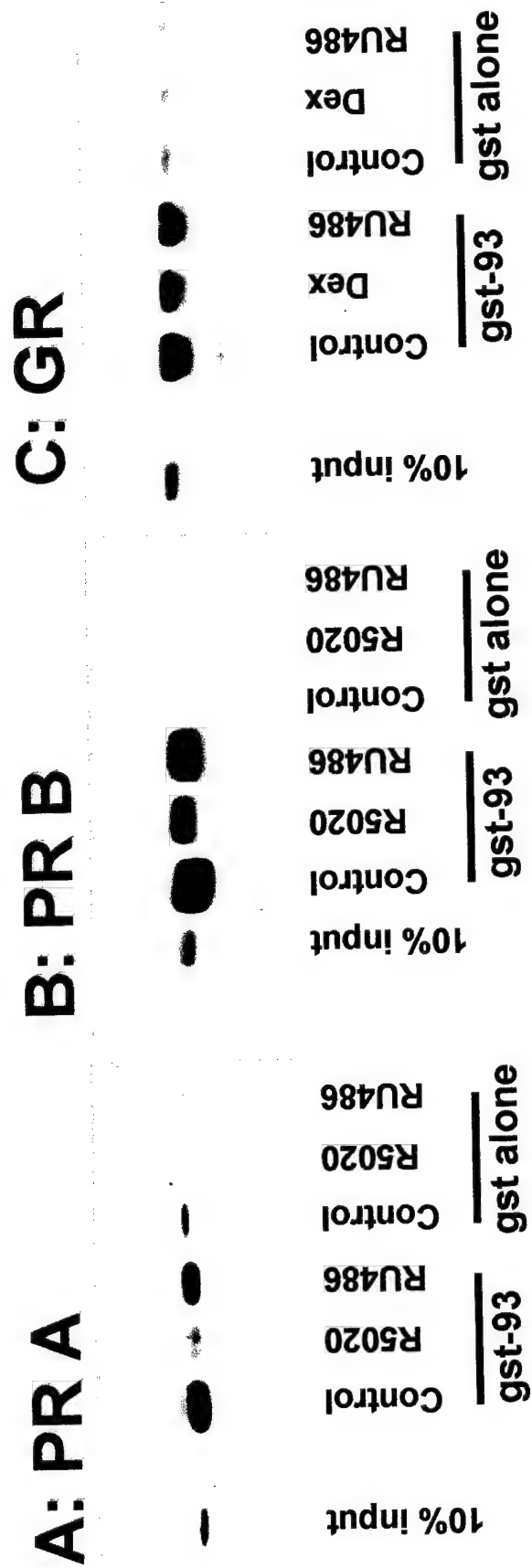


Figure 4

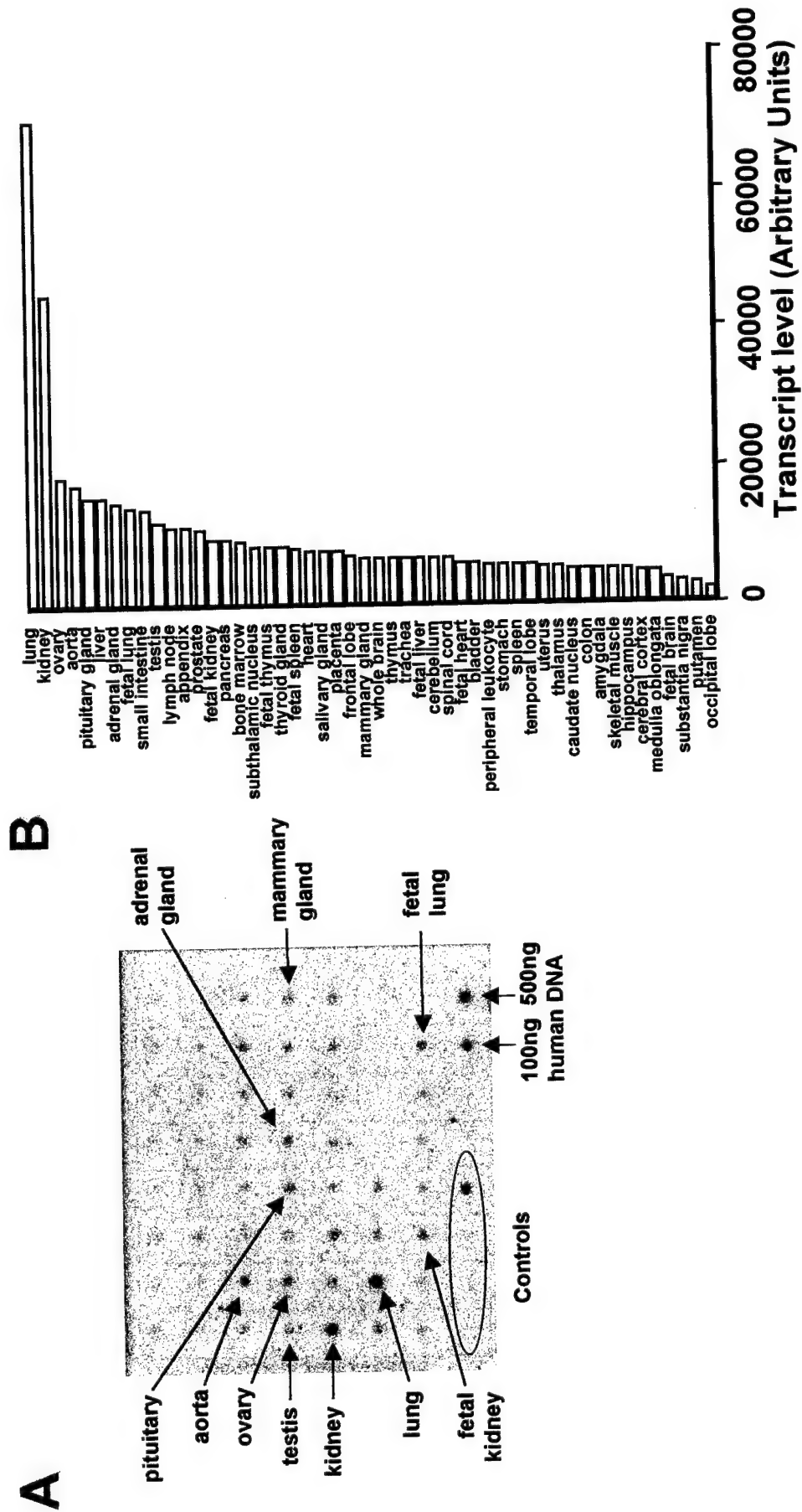


Figure 5

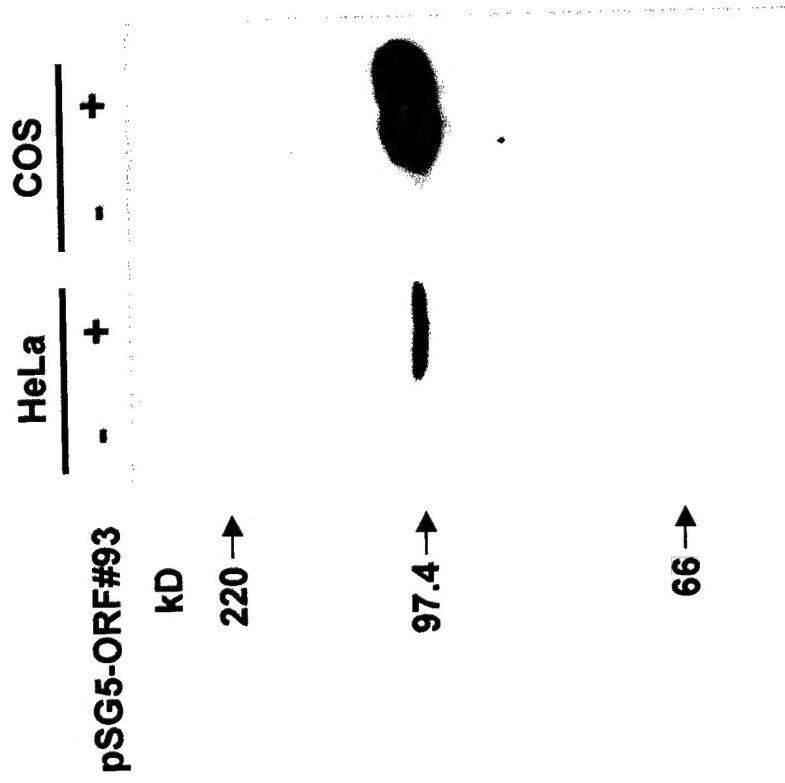


Figure 6

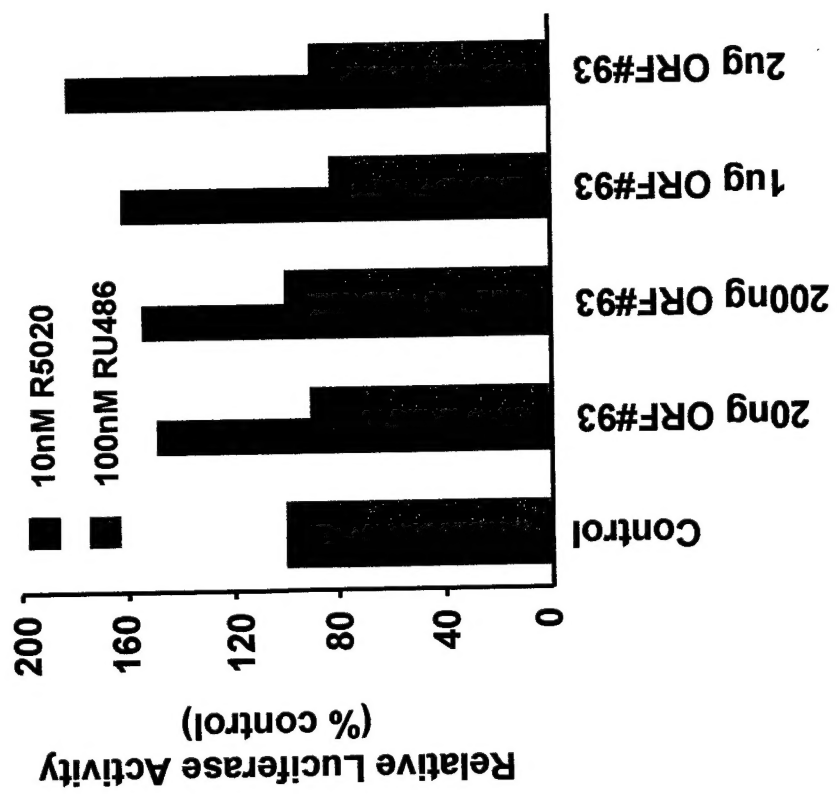


Figure 7

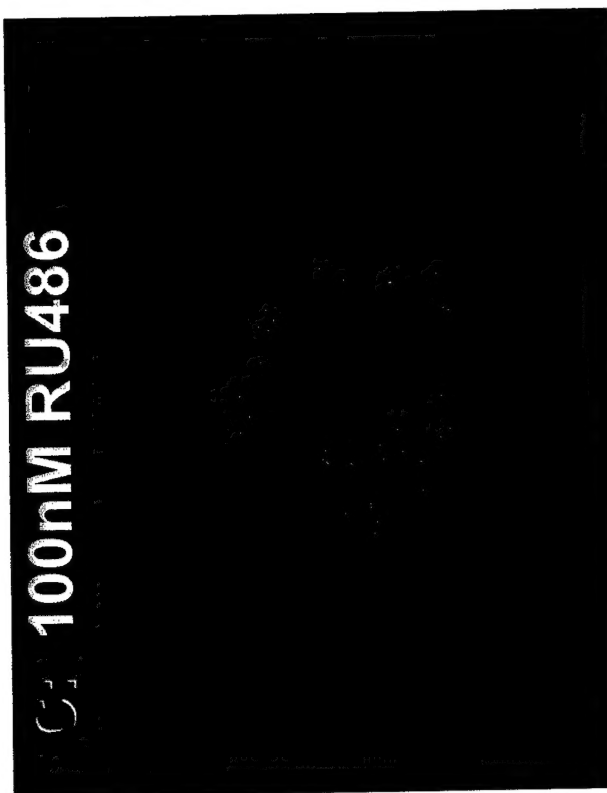
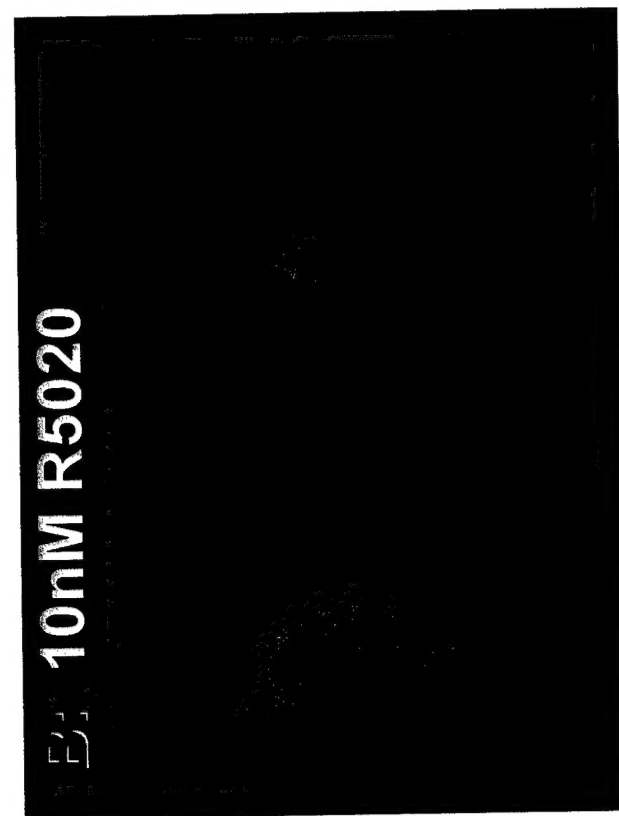
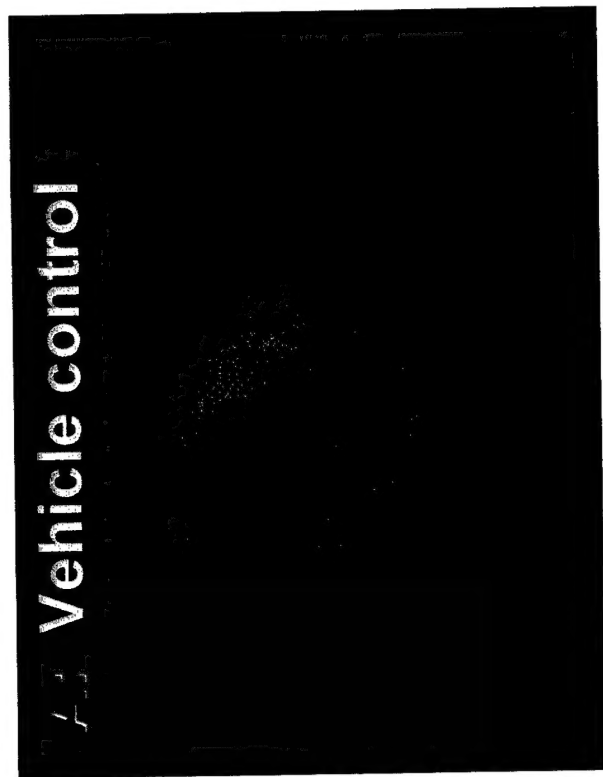


Figure 8

